

Exploring alternative hapten tethering sites for high-affinity anti-picoxystrobin antibody generation

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Abstract

The relevance of the linker tethering site in haptens was investigated for antibody generation and immunoassay development. Three derivatives of the strobilurin fungicide picoxystrobin were synthesized with the same functionalized spacer arm located at three different positions. Protein conjugates of those haptens were employed as immunogens and novel polyclonal antibodies were produced and characterized. All haptens afforded highly specific antibodies, but different affinities to the free analyte were observed among the obtained antisera. Then, competitive enzyme-linked immunosorbent assays were studied in several formats, and site heterology was confirmed as an effective strategy for detectability improvement. An indirect heterologous immunoassay was eventually selected and optimized, showing a limit of detection for picoxystrobin of 0.02 µg/L and a working range between 0.03 and 1.30 µg/L. Finally, the developed extraction and analytical procedures revealed a practical limit of quantification of 5 µg/kg for this fungicide in soybean sprouts, well below the maximum residue limits in the EU.

Keywords: Pesticide; Fungicide; Strobilurin; Hapten synthesis; Polyclonal antibody; ELISA; Immunoassay; Soybean analysis

Introduction

Pesticide analysis is traditionally carried out by instrumental chromatographic separation methods. Alternatively, bioanalytical techniques such as the competitive immunoassays have been revealed as complementary tools for the analysis of chemical residues and contaminants in clinical, food, and environmental samples. Due to the haptenic character of such analytes they need to be coupled to a carrier protein in order to trigger an immunological response. Thus, preparing target derivatives is usually an essential requirement if the generation of high-quality antibodies is sought. A typical synthetic hapten consists of the target molecule and a linker arm containing a functional chemical group for carrier coupling. Therefore, accurate hapten design is a key step for the generation of high-affinity antibodies with the optimal selectivity. Several aspects are believed to influence the properties of the resulting antibodies; for instance, the position and composition of the linker and the preservation of the electronic and steric properties of the target molecule are among the most relevant constraints. However, the optimal parameters may change from one compound to another, so the preparation of a battery of functionalized haptens of the target analyte is certainly recommended. Moreover, the availability of different derivatives makes possible the development of enzyme-linked immunosorbent assays (ELISA)¹, not only with protein conjugates carrying a homologous hapten (the same molecule that was used in the immunogen) but also using a heterologous one, which is a common strategy to improve immunoassay detectability [1,2]. Of all sorts of heterologies, site-heterology, based on a different tethering site of the spacer arm, has been demonstrated as a very promising strategy for achieving high-sensitivity ELISAs, mainly in polyclonal antibody-based assays [3,4].

Strobilurins are a new class of modern synthetic fungicides whose biological activity is due to a pharmacophore group similar to that of the natural active principle (strobilurin A) produced by the fungus *Strobilurus tenacellus*. These pesticides, as compared with other common fungicide groups, show a novel mode of action that is based on the inhibition of mitochondrial respiration by binding to cytochrome b [5]. Among strobilurins, picoxystrobin (PC) shows a particular behavior because it is rapidly absorbed into the plant tissue and it is the most xylem-systemic of all members of this group. For that reason, it is targeted as an early-season application on cultures with preventive and curative purposes. PC can be transferred by air, so the product remaining at the outside of the leaf can be redistributed towards new emerging shoots [6]. This pesticide was approved in the EU in 2003 [7] and it has been registered at the US Environmental Protection Agency as a new chemical candidate [<http://www.epa.gov/opprd001/workplan/newchem.html>]. Nowadays, it is distributed by DuPont as a broad-spectrum fungicide for treatment of cereal grains and oilseed crops [<http://www2.dupont.com>].

Lately, a few papers have been published concerning the development of analytical strategies for PC determination, based on gas chromatography coupled to mass spectrometry or liquid chromatography with diode array detectors [8–10]. Besides, we recently produced an antibody to PC with an affinity value in the low nanomolar range [11]. That antibody was produced by immunization with a protein conjugate of a novel hapten molecule (hapten PCa6) prepared in our lab. This hapten incorporated a six-carbon spacer arm through a carbon–carbon single bond at the aryl ring of the molecule, in the *para* position with respect to the β -methoxyacrylate pharmacophore group (see Table 1). Now, two additional PC derivatives have been synthesized and new polyclonal antibodies (pAbs) have been generated by immunization of rabbits using immunogens of the three synthetic

haptens in order to study the relationship between hapten derivatization site and antibody affinity. The objectives of the present study also comprised the development of immunoassays using the conjugate-coated indirect competitive ELISA (cELISA), the antibody-coated direct cELISA, and the capture-antibody direct cELISA formats, with both homologous and heterologous antigens. The most sensitive assay was fully characterized, optimized, and ultimately applied to the determination of PC residues in soybean sprouts as a relevant commodity for this fungicide.

Materials and methods

Reagents and instrumentation

Solvents and all other reagents used for hapten synthesis were obtained from commercial sources and employed without further purification. *tert*-Butyl 6-bromohexanoate (**16**) was obtained by esterification of 6-bromohexanoic acid with isobutene following a procedure previously described for the preparation of related *tert*-butyl esters [12]. 1-Bromo-2-(bromomethyl)benzene (**7**), *tert*-butyl hex-5-ynoate (**9**), and (*Z*)-methyl 2-iodo-3-methoxyacrylate (**13**) were obtained from 2-bromotoluene, hex-5-ynoic acid, and (*E*)-methyl 3-methoxypropenoate, respectively, according to published procedures [13–15]. Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. NMR spectra were recorded in CDCl₃ or C₆D₆ at room temperature (rt) on a Bruker AC-300 spectrometer (300.13 MHz for ¹H and 75.47 MHz for ¹³C). The spectra were referenced to residual solvent protons in the ¹H NMR spectra (7.26 and 7.15 ppm) and to solvent carbons in the ¹³C NMR spectra (77.0 and 128.02 ppm). Carbon substitution degrees were established by distortionless enhancement by polarization transfer pulse sequences. A combination of correlation spectroscopy and heteronuclear single quantum coherence

experiments was utilized for the assignment of ^1H and ^{13}C chemical shifts. IR spectra were measured as KBr pellets or between NaCl plates using a Nicolet Avatar 320 spectrometer. MS and high resolution mass spectra (HRMS) were obtained by electron-impact (EI) at 70 eV with a Micromass VG Autospec spectrometer. The reactions were monitored with the aid of thin-layer chromatography using 0.25 mm precoated silica gel plates. Chromatography refers to flash column chromatography and it was carried out with the indicated solvents on silica gel 60 (particle size 0.040–0.063 mm).

Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were used for conjugate purification. Horseradish peroxidase (HRP), ovalbumin (OVA), and *o*-phenylenediamine were purchased from Sigma-Aldrich (Madrid, Spain). Polyclonal goat anti-rabbit immunoglobulin (GAR) was from Rockland (Gilbertsville, PA, USA). Polyclonal goat anti-rabbit immunoglobulin peroxidase conjugate (GAR–HRP) was from Bio-Rad (Madrid, Spain). Bovine serum albumin (BSA) fraction V was purchased from Roche Applied Science (Mannheim, Germany). Fetal bovine serum (FBS) and Freund's adjuvants were from Sigma-Aldrich (Madrid, Spain). Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Hapten and conjugate UV–vis spectra and ELISA absorbances were read in dual wavelength mode (492–650 nm) with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments.

PC (methyl (*E*)-3-methoxy-2-{2-[6-(trifluoromethyl)-2-pyridyloxymethyl]phenyl}acrylate, CAS Registry No. 117428-22-5, MW 367.32 g/mol) and other employed pesticide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). Pesticide stock solutions were prepared as concentrated solutions in *N,N*-dimethylformamide (DMF) and kept at $-20\text{ }^{\circ}\text{C}$ in amber glass vials. The composition,

concentration, and pH of the buffers employed in this study were as follows: i) PB, 100 mM sodium phosphate buffer, pH 7.4; ii) PBS, 10 mM sodium phosphate buffer, pH 7.4, with 140 mM NaCl; iii) PBST, PBS containing 0.05% (v/v) Tween 20; iv) PBST-FBS, PBST containing 10% (v/v) FBS; v) PBT-FBS, 200 mM sodium phosphate buffer, pH 7.4, containing 0.05% (v/v) Tween 20 and 10% (v/v) FBS; vi) CB, 50 mM carbonate–bicarbonate buffer, pH 9.6; vii) Washing solution, 150 mM NaCl and 0.05% (v/v) Tween 20; and viii) Developing buffer, 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4.

Hapten synthesis

The synthesis of hapten PCa6 (**1**) was described in a previous article [11] and its structure is schematized in Table 1. Haptens PCb6 (**2**) and PCo6 (**3**) were prepared from 2-chloro-6-trifluoromethyl pyridine (**4**) or PC, respectively, following the synthetic routes depicted in Figs. 1 and 2. A detailed description of each synthetic step as well as the complete spectroscopic characterization data of haptens PCb6 and PCo6 are reported below. For spectroscopic data of all synthetic intermediates see the Supplementary Data.

Synthesis of hapten PCb6 (2)

Synthesis of 2-chloro-4-iodo-6-(trifluoromethyl)pyridine (5). A solution of 2-chloro-6-(trifluoromethyl)pyridine (**4**) (1.533 g, 8.44 mmol) in 2 mL tetrahydrofuran (THF) was added drop wise to a solution of lithium diisopropylamide (LDA) [prepared from 1.6 M butyllithium in hexane (10.5 mL, 16.80 mmol) and diisopropylamine (2.36 mL, 1.704 g, 16.84 mmol) in THF (13 mL)] at –100 °C. After 2 h at this temperature, a solution of I₂ (2.14 g, 8.43 mmol) in THF (2 mL) was slowly added (30 min) to the orange reaction mixture, and then the mixture was allowed to warm to –80 °C for 1 h, poured into water, and extracted with

CH₂Cl₂. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, and concentrated to leave a residue that was purified by chromatography, using hexane–EtOAc (9:1) as eluent, to give iodo-pyridine **5** (1.768 g, 70%) as a colorless solid [mp 94–94.5 °C (crystallized from hexane)], which had physical and spectroscopic properties identical to those reported in the literature [16].

Synthesis of 4-iodo-6-(trifluoromethyl)pyridin-2-ol (6). A mixture of chloro-pyridine **5** (802 mg, 2.6 mmol) and KOH (357 mg, 6.37 mmol) in *tert*-butanol (2.2 mL) was stirred at reflux under nitrogen for 2 h. The mixture was diluted with water, acidified with 1 M aqueous HCl, and extracted with EtOAc. Washing of the extracts with brine was followed by drying over anhydrous Na₂SO₄ and removal of the solvent. Chromatographic purification using mixtures of hexane and EtOAc (from 9:1 to 7:3) as eluent, afforded the 2-pyridinol **6** (621 mg, 63%) as a white solid. Mp 147–148 °C (crystallized from hexane).

Synthesis of 2-(2-bromobenzyloxy)-4-iodo-6-(trifluoromethyl)pyridine (8). A mixture of pyridinol **6** (758 mg, 2.62 mmol) and anhydrous K₂CO₃ (579 mg, 4.19 mmol) in dry DMF (3 mL) was stirred at 80 °C under nitrogen for 5 min, and then a solution of dibromide **7** (681 mg, 2.72 mmol) in DMF (3 mL) was added drop wise by a syringe pump during 4 h. After this time, the reaction mixture was cooled down to rt, poured into water, and extracted with EtOAc. The organic phase was washed with brine, dried over Na₂SO₄, and the residue left after evaporation of the solvent was purified by chromatography, using hexane–EtOAc (95:5), to yield the benzyl ether **8** (930 mg, 78%) as a solid. Mp 67–68 °C (crystallized from hexane).

Synthesis of tert-butyl 6-(2-(2-bromobenzyloxy)-6-(trifluoromethyl)pyridin-4-yl)hex-5-ynoate (10). Et₃N (2.8 mL) was added drop wise to a mixture of iodo pyridine **8** (480 mg, 2.139 mmol), *tert*-butyl hex-5-ynoate (**9**) (378 mg, 2.351 mmol), Cl₂Pd(PPh₃)₂ (36.6 mg,

0,052 mmol), and CuI (7.6mg, 0.040 mmol) in anhydrous DMF (2.8 mL) under nitrogen. The mixture was stirred at rt for 5 h, poured into water (100 mL), and extracted with EtOAc. The combined organic extracts were washed successively with water, 2% aqueous solution of LiCl, and brine, dried over anhydrous Na₂SO₄ and concentrated. Purification by chromatography, using hexane–EtOAc (9:1) as eluent, afforded alkyne **10** (916 mg, 86%) as a colorless oil.

Synthesis of tert-butyl 6-(2-(2-bromobenzyloxy)-6-(trifluoromethyl)pyridin-4-yl)hexanoate (11). A solution of alkyne **10** (882 mg, 1.783 mmol) and chlorotris(triphenylphosphine)rhodium(I) (100 mg, 0.108 mmol, 6%) in anhydrous THF (10.5 mL) was evacuated and purged under an atmosphere of hydrogen gas. The hydrogen pressure was regulated to 4 bars and the reaction mixture was stirred at rt for 21 h. Then, the solvent was removed under vacuum and the obtained residue was purified by chromatography, using hexane–EtOAc (9:1) as eluent, to give compound **11** (1.049 g, 92%) as a colorless oil.

Synthesis of tert-butyl 6-(2-(2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyloxy)-6-(trifluoromethyl)pyridin-4-yl)hexanoate (12). Et₃N (843 µL, 0.037 mmol) was added to a mixture of aryl bromide **11** (760 mg, 1.513 mmol), Pd(OAc)₂ (8.5 mg, 0.037 mmol), and 2-dicyclohexylphosphinobiphenyl (53 mg, 0.151 mmol) in dry dioxane (3.6 mL) under nitrogen. After stirring at rt for a few minutes, pinacolborane (548 µL, 3.782 mmol) was added and the mixture was stirred at 80 °C for 3.5 h. The reaction mixture was cooled to rt and quenched by the addition of an aqueous saturated solution of NH₄Cl, then diluted with water, and extracted with EtOAc. The combined organic extracts were washed with brine, dried over Na₂SO₄, and concentrated. The obtained residue was purified by

chromatography, using hexane–EtOAc (95:5) as eluent, to yield the boronic acid ester **12** (493 mg, 60%) as an oil.

Synthesis of (E)-tert-butyl 6-(2-(2-(1,3-dimethoxy-3-oxoprop-1-en-2-yl)benzyloxy)-6-(trifluoromethyl)pyridin-4-yl)hexanoate (14). A mixture of arylboronic acid **12** (472 mg, 0.861 mmol), iodoacrylate **13** (312 mg, 1.291 mmol), K₃PO₄ (548 mg, 2.583 mmol), and Pd(PPh₃)₄ (39.8 mg, 0.034 mmol) in a mixture of dioxane (3.6 mL) and water (0.7 mL), previously degasified by bubbling nitrogen under ultrasonic irradiation for 10 min, was stirred under nitrogen at 95 °C for 10 h and then at 100 °C for an additional 2 h. The reaction mixture was cooled down to rt, diluted with ethyl ether, washed with brine, and dried over anhydrous Na₂SO₄. Evaporation of the solvent and chromatographic purification of the residue, using hexane–EtOAc (9:1) as eluent, gave the methoxyacrylate derivative **14** (296 mg, 64%) as an oil.

Synthesis of (E)-6-(2-(2-(1,3-dimethoxy-3-oxoprop-1-en-2-yl)benzyloxy)-6-(trifluoromethyl)pyridin-4-yl)hexanoic acid (hapten PCb6, 2). A solution of the *tert*-butyl ester **14** (101 mg, 0.188 mmol) in formic acid (3.5 mL) was stirred for 2 h at rt under nitrogen. The reaction mixture was diluted with benzene and washed four times with cool water and once with brine, dried over anhydrous Na₂SO₄, and concentrated under vacuum to give the crude product that was purified by chromatography, using CHCl₃ as eluent, to give hapten PCb6 (**2**) (67 mg, 75%) as a viscous oil. ¹H NMR (C₆D₆), δ: 7.59 (1H, m, H-6 Ph), 7.41 (1H, s, =CH), 7.29 (1H, m, H-3 Ph), 7.17–7.10 (2H, m, H-4 Ph and H-5 Ph), 6.83 (1H, br s, H-5 Py), 6.48 (1H, br s, H-3 Py), 5.60 (1H, s, CH₂O), 3.44 (3H, s, CO₂Me), 2.91 (2H, s, OMe), 2.01 (2H, t, *J* = 7.3 Hz, H-6), 1.96 (2H, t, *J* = 7.8 Hz, H-2), 1.34 (4H, quint, *J* = 7.3 Hz, H-5), 1.06 (3H, m, H-3), 0.91 (2H, m, H-4); ¹³C NMR (C₆D₆), δ: 179.87(C-1), 167.88 (CO₂H), 164.62 (C-2 Py), 160.10 (C=CH), 155.71 (C-4 Py), 145.46 (q, *J* = 34 Hz, C-6 Py), 136.41 (C-1 Ph), 133.18(C-2 Ph), 131.69 (C-3

Ph), 128.97 (C-6 Ph), 127.92 and 127.87 (C-4 Ph and C-5 Ph), 122.52 (q, $J = 274$ Hz, CF_3), 114.36 (q, $J = 3$ Hz, C-5 Py), 114.09 (C-3 Py), 110.79 (C=CH), 66.93 (CH_2O), 60.91 (OMe), 51.22 (CO_2Me), 34.66 (C-2), 33.80 (C-6), 29.43 (C-3), 28.45 (C-4), 24.41 (C-5); IR (NaCl), ν_{max} : 3500–2500, 3060, 3033, 2946, 1708, 1622, 1567, 1437, 1400, 1350, 1259, 1187, 1137, 1039, 771, 733 cm^{-1} ; MS (EI), m/z (%): 481 (M^+ , 7), 450 (4), 449 (17), 205 (21), 204 (31), 191 (8), 190 (66), 189 (14), 175 (16), 173 (18), 145 (100); HRMS: calculated for $\text{C}_{24}\text{H}_{26}\text{F}_3\text{NO}_6$ 481.17122, found 481.17171. UV (PB), ϵ ($\text{mM}^{-1} \text{cm}^{-1}$): (280 nm) 2.81, (260 nm) 8.37.

Synthesis of hapten PCo6 (**3**)

Synthesis of (*E*)-methyl 3-hydroxy-2-(2-((6-(trifluoromethyl)pyridin-2-yl)oxy)methyl)phenyl)acrylate (**15**). PC (518 mg, 1.410 mmol) was added in small portions to a well stirred solution of $\text{LiOH}\cdot\text{H}_2\text{O}$ (593 mg, 14.10 mmol) and Bu_4NHSO_4 (479 mg, 1.410 mmol) in a 3:1 mixture of THF– H_2O (14 mL). The mixture was stirred at rt for 16 h, then diluted with H_2O (50 mL), acidified to pH 3–4 with solid KHSO_4 and extracted with ethyl ether. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 , and concentrated to dryness. The residue was purified by silica gel chromatography, using hexane–EtOAc (9:1) as eluent, to give enol **15** (408 mg, 82%) as an oil [17].

Synthesis of (*E*)-tert-butyl 6-(3-methoxy-3-oxo-2-(2-((6-(trifluoromethyl)pyridin-2-yl)oxy)methyl)phenyl)prop-1-en-1-yl)hexanoate (**17**). A mixture of enol **15** (390 mg, 1.104 mmol) and anhydrous Cs_2CO_3 (443 mg, 1.359 mmol) in dry DMF (10 mL) was stirred at rt for 15 min. *tert*-Butyl 6-bromohexanoate (**16**, 554 mg, 2.208 mmol) was added and the mixture was stirred for 2 h, then diluted with H_2O (50 mL), and extracted with EtOAc. The combined organic extracts were successively washed with 2% aqueous solution of LiCl and brine, dried

over anhydrous MgSO_4 , and concentrated. The residue was chromatographed on silica gel, using hexane–EtOAc (9:1) as eluent, to give the enol ether **17** (474 mg, 82%) as an oil.

Synthesis of (E)-6-(3-methoxy-3-oxo-2-(2-((6-(trifluoromethyl)pyridin-2-yloxy)methyl)phenyl)prop-1-enyloxy)hexanoic acid (hapten PCo6, **3**). A solution of *tert*-butyl ester **17** (312 mg, 0.597 mmol) in dry CH_2Cl_2 (1 mL) was treated with $\text{CF}_3\text{CO}_2\text{H}$ (1 mL) under nitrogen at 0 °C. The mixture was stirred at the same temperature for 2 h, then diluted with benzene, washed with brine, dried over anhydrous Na_2SO_4 , and concentrated to dryness in vacuum. The residue was purified by chromatography, using CHCl_3 –MeOH (99:1) as eluent, to give hapten PCo6 (270 mg, 82%) as a viscous oil. ^1H NMR (CDCl_3), δ : 7.68 (1H, dd, J = 8 and 8 Hz, H-4 Py), 7.62 (1H, s, =CH), 7.56 (1H, m, H-3 Ph), 7.33 (2H, m, H-4 Ph and H-5 Ph), 7.23 (1H, br d, J = 8 Hz, H-5 Py), 7.16 (1H, m, H-6 Ph), 6.89 (1H, br d, J = 8 Hz, H-3 Py), 5.31 (2H, s, CH_2O), 4.00 (2H, t, J = 7 Hz, H-6), 3.67 (3H, s, CO_2Me), 2.93 (2H, t, J = 7 Hz, H-2), 1.69–1.49 (4H, m, H-3 and H-5), 1.33 (2H, m, H-4); ^{13}C NMR (CDCl_3), δ : 179.22 (C-1), 168.22 (CO_2Me), 163.57 (C-2 Py), 158.97 (C=CH), 145.25 (q, J = 34 Hz, C-6 Py), 139.30 (C-4 Py), 135.50 (C-2 Ph), 132.21 (C-1 Ph), 131.03 (C-6 Ph), 128.63 (C-3 Ph), 127.71 (C-4 Ph and C-5 Ph), 121.33 (q, J = 274 Hz, CF_3), 114.62 (C-3 Py), 113.21 (q, J = 3 Hz, C-5 Py), 109.74 (C=CH), 74.86 (C-6), 66.31 (CH_2O), 51.56 (CO_2Me), 33.66 (C-2), 29.23 (C-5), 24.85 (C-4), 24.03 (C-3); IR (NaCl), ν_{max} : 3100, 2951, 1708, 1627, 1458, 1350, 1285, 1143, 989, 811, 752 cm^{-1} ; MS (EI), m/z (%): 467 (11, M^+), 436 (27), 435 (100), 336 (17), 335 (85), 303 (69), 190 (49), 158 (68), 131 (83), 103 (65); HRMS: calculated for $\text{C}_{23}\text{H}_{24}\text{F}_3\text{NO}_6$ 467.15557, found 467.15498. UV (PB), ϵ ($\text{mM}^{-1}\text{cm}^{-1}$): (280 nm) 3.13, (260 nm) 9.22.

Preparation of protein–hapten conjugates

All conjugates used in this study were prepared by activation of the free carboxylic group of haptens PCa6 (**1**), PCb6 (**2**), and PCo6 (**3**) and reaction with the free amine groups of different carrier proteins: BSA for the immunizing conjugate, OVA for coating of ELISA plates in indirect assays, and HRP as enzyme tracer for the direct formats. On the one hand, immunizing conjugates were prepared by the active ester method *via* an alternative procedure using *N,N'*-disuccinimidyl carbonate (DSC) as activating reagent instead of the classical carbodiimide derivatives, as previously reported [11]. On the other hand, assay conjugates were obtained by the classical mixed anhydride procedure [18]. Three OVA conjugates were prepared from each hapten using decreasing hapten-to-protein molar ratios (MRs) in the reaction mixture (13, 7.5, or 1.3 mole of hapten was added for coupling per mole of protein). If conjugation occurred, the UV–vis spectrum of the conjugate was slightly different from that of the free protein, so the finally achieved hapten-to-protein MR could be measured spectrophotometrically by assuming that the molar absorptions of both the hapten and the protein were the same for the free and the conjugated forms. For further details see the Supplementary Data.

Production of antibodies

Animal manipulation was performed in compliance with laws of the Spanish Ministry of Agriculture, Fisheries, and Food and following European guidelines. Two female New Zealand white rabbits weighing 1–2 kg were immunized by subcutaneous injection with 0.3 mg of BSA–hapten conjugate in 1.0 mL of a 1:1 mixture of conjugate solution in PB and complete Freund's adjuvant. Animals were boosted at 21-day intervals with the same immunogen suspended in a mixture of 0.5 mL of PB and 0.5 mL of incomplete Freund's

adjuvant. Whole blood was collected from the ear vein of the rabbits and by intracardiac puncture 10 days after the fourth injection. Blood samples were allowed to coagulate overnight at 4 °C. Then, the serum was separated by centrifugation and the immunoglobulin fraction was precipitated by adding 1 volume of a saturated ammonium sulfate solution. After centrifugation at $4000 \times g$ for 0.5 h, the pellet was resuspended in 1 volume of PBS and a fraction was diluted 1/5 with PBS containing 0.01% thimerosal (w/v) and kept at 4 °C for daily usage. The remainder of each antiserum was precipitated again as before and stored at 4 °C.

Direct cELISA procedures

Direct assays were performed with and without a capture antibody. Ninety-six-well polystyrene ELISA plates were coated with 100 μ L per well of antibody dilution in CB by overnight incubation at rt. In between steps, plates were washed four times with washing solution. For capture-antibody assays, a coating GAR solution was used, and after washing 100 μ L of anti-PC pAb solution was added per well, then plates were incubated 1 h at rt and washed as before. In both formats, the competitive step was carried out with 50 μ L per well of analyte standard solution in PBS plus 50 μ L per well of HRP tracer dilution in PBST. The immunological reaction took place during 1 h at rt, and plates were washed again as described. Finally, signal was generated by addition of 100 μ L per well of freshly prepared 2 mg/mL *o*-phenyldiamine and 0.012% (v/v) H₂O₂ in developing buffer. After 10 min at rt, the enzymatic reaction was stopped by addition of 100 μ L per well of 2.5 M sulfuric acid. The absorbance was immediately read at 492 nm using a reference wavelength of 650 nm.

Indirect cELISA procedure

Microplates were coated with 100 μ L per well of OVA–hapten solution in CB by overnight incubation at rt. Coated plates were washed four times with washing solution and then received 50 μ L per well of analyte standard solution in PBS plus 50 μ L per well of anti-PC pAb in PBST-FBS. The immunological reaction took place during 1 h at rt, and plates were washed again as described above. Next, 100 μ L per well of a $1/10^4$ dilution of GAR–HRP conjugate in PBST-FBS was added, and then plates were incubated 1 h at rt and washed as before. Finally, the signal was produced as aforementioned.

Buffer studies

The influence of the ionic strength and pH over the inhibition curve parameters was studied using a series of buffers prepared as follows. First, a 40 mM trisodium citrate, 40 mM disodium hydrogenphosphate and 40 mM Tris solution (pH 9.9) was prepared and known volumes of 5 M HCl was added in order to reach the required pH in each case. Then, the ionic strength of all buffers was fixed by adding the appropriate volume of a 2 M NaCl solution and taking into account the initial solution and the added HCl. Finally, Tween 20 was added before the final volume was achieved with deionized water. PC standard curves were prepared in water, and they were mixed with the tracer dilution for direct competitive assays or with the polyclonal solution for indirect competitive assays in every of the prepared buffers. Changes in the PC inhibition curve parameters using distinct buffers were fitted by a multiple regression equation, including curvature and interaction terms, using Minitab 14.1 software (Minitab Inc., State College, PA, USA) as previously published [19].

Analyte extraction

Soybean sprouts were purchased from local markets and short-term stored at 4 °C. Samples were chopped and homogenized using a T-25 ultra-turrax blender from IKA (Staufen, Germany) and if required stored at –20 °C. Extraction of PC from soybean samples was performed by the QuEChERS method [20] using the DisQuE dispersive sample preparation kit from Waters (Milford, MA, USA) without dispersive solid phase extraction. Briefly, 15 g of homogenate was introduced in a 50 mL polypropylene centrifuge tube with 1.5 g of NaOAc, 6 g of MgSO₄, and 15 mL of 1% HOAc in acetonitrile. Tubes were vigorously shaken using a vortex mixer and then centrifuged at 2200 × g for 5 min. The organic extracts were analyzed by the optimized indirect cELISA after being properly diluted with deionized water.

Data treatment

Calibration standards were prepared by 6-fold serial dilution from a 1 mM PC stock solution in DMF. Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Assay detectability was estimated as the concentration of analyte at the inflection point of the sigmoidal curve, typically corresponding to a 50% inhibition (IC₅₀) of the maximum absorbance reached at the zero dose of analyte (A_{\max}) if the background signal approaches to zero. The limit of detection (LOD) was estimated as the concentration of PC that provided a 10% inhibition of A_{\max} , whereas the analytical working range was calculated as the PC concentration that provided from 20 to 90% inhibition of A_{\max} .

Results and discussion

Haptens synthesis and conjugate preparation

In this study, three haptens, namely PCa6 (**1**), PCb6 (**2**), and PCo6 (**3**) (Table 1), were used for antibody generation and immunoassay development. The strategy for the preparation of PCb6 (**2**) was based on that previously reported for hapten PCa6 (**1**) [11] and for some functionalized derivatives of the related strobilurin fungicide azoxystrobin [21]. Such approach relies on the different reactivity of aryl iodide and bromide groups towards palladium-catalyzed Sonogashira and Suzuki-Miyaura cross-coupling reactions, as key steps for the modular incorporation of the hydrocarbon spacer chain and the β -methoxyacrylate moiety, respectively. The designed synthetic scheme for hapten PCb6, summarized in Fig. 1, required the initial preparation of a fragment containing the pyridine and phenyl moieties (intermediate **8**), which was synthesized starting with iodination of the C-4 position of 2-chloro-6-trifluoromethyl pyridine (**4**) and, following the Schlosser protocol [16], it was transformed into 4-iodo pyridine **5**. Replacement of the chlorine atom by a hydroxyl group, followed by *O*-alkylation of the thus-formed 2-pyridinol **6** with 1-bromo-2-(bromomethyl)benzene (**7**) catalyzed by K_2CO_3 led to the required benzyl ether **8**. This compound underwent a chemoselective and efficient palladium-catalyzed Sonogashira reaction with *tert*-butyl hex-5-ynoate (**9**) to give the acetylenic compound **10**. Such cross-coupling reaction was very selective and no substantial reaction at the phenyl-brominated position was observed. Homogeneous catalyzed hydrogenation of the triple bond of **10** using Wilkinson's catalyst afforded the C-4 alkylated pyridine derivative **11**, thus completing the introduction of the C6 hydrocarbon chain that constituted the spacer arm of this hapten. Introduction of the β -methoxyacrylate moiety that completed the PCb6 hapten

framework was achieved through a Suzuki-Miyaura reaction. First, the aryl bromide **11** was converted to the arylboronic ester **12** by treatment with pinacolborane and Pd(OAc)₂/2-(dicyclohexylphosphino)biphenyl in Et₃N–dioxane, which was then subjected to a second Pd-catalyzed cross-coupling reaction with methyl (Z)-2-iodo-3-methoxyacrylate (**13**) to yield the β-aryl-β-methoxyacrylate **14** in a moderate 40% overall yield from the starting aryl bromide **11**. Finally, chemoselective formic acid-promoted hydrolysis of the *tert*-butyl ester moiety of **14** afforded hapten PCb6 (**2**) in good yield.

Hapten PCo6 (**3**) was readily prepared from commercial PC through a three-step procedure (Fig. 2). First, the β-methoxyacrylate moiety of PC was hydrolyzed to the corresponding enol (**15**) by treatment with LiOH and Bu₄NHSO₄ in a mixture of THF–water. Despite the strong basic medium that was employed, the reaction took place quite cleanly under these conditions, probably through Michael addition of the hydroxyl anion followed by elimination of the methoxyl group, with only a small amount (*ca.* 10%) of the carboxylic acid being formed by hydrolysis of the methyl ester moiety. Next, the spacer group was covalently bound to the hydroxyl moiety of **15** via *O*-alkylation reaction with *tert*-butyl 6-bromohexanoate (**16**) and Cs₂CO₃ as basic catalyst in DMF to give the enol ether **17**, whose *tert*-butyl ester moiety was chemoselectively hydrolyzed to the corresponding acid by treatment with formic acid at rt. Hapten PCo6 (**3**) was thus obtained in about 55% overall yield from starting PC.

The three haptens (PCa6, PCb6, and PCo6) incorporated the same spacer arm, a six carbon-atom linker consisting of a linear aliphatic saturated chain, attached to three rationally-selected tethering sites of the PC molecule. In hapten PCa6, the linker was anchored to the central aryl bridge that is present in all synthetic strobilurins; in hapten PCb6, the derivatization site was located at the characteristic moiety of PC; and in PCo6, the

arm was introduced at the β -methoxyacrylate pharmacophore group. A three dimensional representation of one of the energetically-equivalent most stable conformations of PC showed that the three selected derivatization sites allowed the target molecule to be displayed from scattered angles (Fig. 3). Moreover, these synthetic haptens preserved the entire skeleton and functional groups of PC, and in all cases a C–C covalent bond replacing a C–H chemical bond was formed between the molecule and the spacer. Therefore, no or minor modifications of steric and electronic features of the target compound were expected.

Immunizing conjugates were prepared by the active ester method from the purified *N*-succinimidyl ester of the hapten. Activation of the carboxyl moiety was easily achieved with high yields (80%) using DSC as described in the Supplementary Data. Purification of the active ester could be accomplished without difficulty by chromatography on silica gel because no significant by-products were formed during the activation reaction. After coupling, the conjugate was purified by gel filtration and the calculated hapten-to-protein MRs of the BSA conjugates were 16, 13, and 20 for PCa6, PCb6, and PCo6 conjugates, respectively. On the other hand, assay conjugates were prepared by the classical mixed anhydride method. Three OVA conjugates were prepared with different amounts of each hapten, and the calculated final MRs were 6, 3, and 1, for the OVA–PCa6 conjugates, 5, 3, and 1 for the OVA–PCb6 conjugates, and 4, 2, and 1 for the OVA–PCo6 conjugates.

Assay format and immunoreagent selection

Two pAbs were obtained with every immunizing hapten; namely, antisera rPCa6#1 and rPCa6#2 were produced from hapten PCa6, antisera rPCb6#1 and rPCb6#2 from PCb6, and antisera rPCo6#1 and rPCo6#2 from the PCo6-based immunogen. Combinations of all

antisera and all conjugates were evaluated using the direct and indirect cELISA formats. Checkerboard direct competitive assays were performed by coating with antiserum dilutions (from 1/1000 to 1/30000) in CB, and mixing tracer solutions (from 30 to 1000 ng/mL) in PBST with a complete PC standard curve in PBS. Very low absorbance signals were obtained under these conditions (results not shown), suggesting either a low recognition of tracer conjugates or a loss of antibody activity due to the coating process. Antibody inactivation due to direct immobilization on polystyrene plates can often be avoided by pre-coating the microwells with a capture bioreceptor [22,23]. Accordingly, antisera were evaluated in the direct cELISA format in wells pre-coated with an anti-rabbit immunoglobulin antibody. In this so-called capture-antibody cELISA format, GAR-coated plates received different dilutions of anti-PC antiserum in PBST, and checkerboard competitive assays were performed as aforementioned. With this procedure, antisera binding properties partially increased, although in order to obtain adequate maximum signals the required concentration of immunoreagents (antisera and/or enzyme tracers) were mostly unusually high (Table 2). All three immunogens generated high-affinity antibodies, with IC_{50} values in the low part-per-billion range. However, assays with IC_{50} values below 1 ng/mL were only reached with antisera derived from the PCo6-based immunogen, due perhaps to the higher degrees of freedom of the β -methoxyacrylate moiety. It was also observed that antibodies obtained with BSA-PCb6 only recognized the homologous antigen; likewise, the tracer HRP-PCb6 was only bound by PCb6-type antibodies. However, antibodies derived from PCa6 could generally bind the tracer of PCo6, and *vice versa*; that is, PCo6-type antibodies also recognized HRP-PCa6. It seems that PCa6- and PCo6-based immunogens generated antibodies with a binding pocket in which the pyridyl ring of the PC molecule played a determinant role, whereas antisera obtained by immunization with BSA-PCb6 did not

accept antigens with the tethering site at the aryl ring (PCa6) or at the pharmacophore group (PCo6). Similar results have also been found with antisera generated against site-heterologous immunogens of azoxystrobin (unpublished results) and pyraclostrobin [24].

Checkerboard competitive titration assays were carried out as well in the conjugate-coated indirect ELISA format. Plates were coated with OVA-PCa6 (MR = 6), OVA-PCb6 (MR = 5), and OVA-PCo6 (MR = 4) at two concentrations (100 and 1000 ng/mL) in CB, and the immunological reaction was performed with four antibody dilutions (from 1/3000 to 1/100000) in PBST-FBS and PC standard solutions in PBS. In this format, a set of inhibition curves could be obtained for most pairs of immunoreagents, reaching variable A_{max} values. Table 3 lists, for each antibody-antigen combination, the representative parameters of that particular inhibition curve displaying the lowest IC_{50} and an A_{max} above 0.8. This cELISA format tolerated more heterologous combinations than the direct assays. Nevertheless, like in the previous format, no assay could be obtained with PCa6-type antisera combined with the coating conjugate of hapten PCb6. Regarding detectability, the IC_{50} values for PCb6- and PCo6-derived pAbs were below 3.60 ng/mL, whereas PCa6-based immunogens afforded antibodies with the lowest affinity to PC. However, a proper heterologous antigen, such as OVA-PCo6, could help to achieve good detectability with medium-affinity antisera, like rPCa6#1 and rPCa6#2. The best immunoassay was achieved with pAb rPCo6#1 and conjugate OVA-PCa6. This result could be explained by the nearer position of the spacer arm in haptens PCo6 and PCa6 as compared with the tethering site in hapten PCb6 (Fig. 3).

Cross-reactivity studies were performed in order to find other pesticides that could be recognized by the produced pAbs. Consequently, eight strobilurin fungicides (kresoxim-methyl, trifloxystrobin, pyraclostrobin, azoxystrobin, dimoxystrobin, fluoxastrobin, metominostrobin, and orysastrobin) were assayed. Calibration curves were prepared up to

1 mg/L in PBS and measured by homologous indirect cELISA. Higher concentrations could not be tested because of the low water solubility of the studied compounds. Under these conditions, none of the produced pAbs showed cross-reactivity values higher than 0.1%. This result indicated that all three immunizing haptens, despite the different site to which the spacer arm was attached, gave rise to equally selective antibodies.

For further ELISA development, a heterologous assay was selected in the conjugate-coated indirect competitive format taking into consideration not only the achieved detectability but also immunoreagent economy. Thus, for sensitive and selective PC detection and analysis, antigen OVA–PCa6 and antiserum rPCo6#1 were employed for microplate coating and for analyte binding, respectively.

Molar ratio of the OVA–hapten conjugates

The effect of reducing the hapten density of the OVA–PCa6 conjugate was evaluated with antibody rPCo6#1. Checkerboard cELISAs were carried out with antigens bearing different hapten-to-protein ratios (5, 3, and 1). Plates were coated with each OVA–PCa6 conjugate at 100, 300, and 1000 ng/mL in CB, and then several rPCo6#1 dilutions (from 1/1000 to 1/30000) in PBST-FBS and a PC standard curve in PBS were added. Table 4 shows the four parameters of the best inhibition curve for every evaluated coating condition. If the antigen with the lower hapten density was used, the A_{\max} values were too low, and raising the antibody concentration made the A_{\min} values to increase. The use of the OVA–PCa6 conjugate with an intermediate hapten density (MR = 3) at 1000 ng/mL together with rPCo6#1 antibody at a 1/10000 dilution, provided more sensitive assays, with a lower antibody consumption, and slightly better background signals than with the antigen of a

higher MR. Therefore, further studies went on with the OVA–PCa6 conjugate of moderate haptenic charge.

Solvent tolerance

Organic solvents are commonly required for pesticide extraction from solid samples. The presence of solvents in the measured sample solution may interfere with the interaction between antibody and analyte, providing an erroneous response. For that reason, changes in the key parameters of the inhibition curve (A_{\max} and IC_{50}) were monitored using PC standards prepared in PBS containing different percentages of methanol, ethanol, acetonitrile, or acetone (Fig. 4). The presence of acetone or acetonitrile in the assay strongly modified the immunological reaction. On the contrary, 10% methanol had a minor effect over the standard curve, whereas ethanol showed an intermediate behavior.

Buffer conditions

The influence of buffer conditions (pH and ionic strength) over the immunoassay was evaluated by a multiparametric strategy. Three buffer systems, *i.e.* citrate ($pK_{a2} = 4.8$, $pK_{a3} = 6.4$), phosphate ($pK_{a2} = 7.2$), and Tris ($pK_a = 8.1$), together with NaCl, were employed to adjust pH and ionic strength values, as described by Abad-Fuentes *et al.* [19]. Tween 20 and FBS were added at a fixed concentration (0.05% and 10%, respectively) in order to reduce unspecific interactions. A full factorial design, including 4 square, 4 axial, and 5 centre points ($n = 3$), was employed with assay pH values from 5.5 to 9.5, ionic strengths from 50 to 300 mM, and PBST-FBS conditions as the center point (see Table S1 of the Supplementary Data). Conjugate-coated cELISAs were carried out using PC standard curves in water, whereas pAb rPCo6#1 was diluted (1/10000) in every evaluated buffer. A_{\max} and IC_{50} were taken as

response values and fitted to a multiple regression equation, including curvature and interaction terms, using the Minitab software.

Significant changes over the inhibition curve parameters were observed upon pH and ionic strength variations (see the respective contour plots in Fig. S1 of the Supplementary Data). The A_{\max} value was maximum at pH close to 7.5 and it decreased at acidic and basic pHs, whereas it decreased at higher ionic strength values. In the case of assay detectability, the lowest IC_{50} was reached at acidic pHs and high NaCl concentrations. When the A_{\max} and IC_{50} contour plots were overlaid (Fig. 5), a constricted area of pH and ionic strength variations was clearly defined in which changes in those parameters remained tolerable; that is, between 80 and 120%. Consequently, in order to guarantee minimum pH variations during food analysis, a more concentrated buffer than PBS was investigated. Fig. 6 shows the sigmoidal curves obtained for the heterologous cELISA using rPCo6#1 and OVA-PCa6 (MR = 3) in two buffer systems with the same pH and equivalent ionic strength but different buffering capacity. Since both buffer systems afforded equivalent results, a more concentrated buffer (PBT-FBS) was selected for antiserum dilution in further studies using food samples. At the end of the whole optimization process, the IC_{50} value of the assay was 0.13 ± 0.01 ng/mL, the calculated LOD was 0.02 ng/mL, and the working analytical range was established between 0.03 and 1.30 ng/mL.

Analysis of PC in spiked soybean sprouts

The final aim of the present study was the development of a bioanalytical system for the determination of PC residues in food. Soybean sprouts were selected as a model oilseed foodstuff, and the well-known multiresidue QuEChERS methodology [25], based on an acetonitrile extraction of acetate-buffered samples, was employed for pesticide recovery.

Sample preparation for ELISA analysis usually consists in a simple dilution of the extracts with assay buffer [26,27]. Accordingly, PC standard curves were prepared in diluted soybean sprout extracts (1/10, 1/30, 1/100, 1/300, and 1/1000 with deionized water) to assess the matrix effects on curve parameters (Fig. 7). An evident reduction in assay signal was observed at extract dilutions lower than 100 fold, so this factor was taken as the minimum required dilution to obtain reliable results.

Trueness and precision of PC determinations were evaluated by measuring QuEChERS-based extracts of soybean sprouts which had been fortified with PC at concentrations ranging from 5 to 1000 µg/kg. Unspiked soybean extracts were also included as negative controls. Table 5 shows the recoveries obtained for PC-spiked samples at three dilution levels (1/100, 1/300, and 1/1000) in order to cover the quantification range of the assay. The precision of the developed ELISA was satisfactory, with relative standard deviation values lower than 20% in all cases. Our results also showed that PC residues in soybean sprouts could be determined by this cELISA with quantitative recoveries (from 92 to 118 %). From this study, the limit of quantification (LOQ) for the optimized immunoassay could be established at 5 µg/kg.

In this study, we observed that the derivatization of the PC molecule at the β -methoxyacrylate moiety generated pAbs with higher affinity. Assay detectability could be improved using a site-heterologous antigen, though application of such a strategy with the direct cELISA format meant binding failure in many cases. The observed results could be explained by a differential display of the PC molecular framework in haptens PCa6, PCb6, and PCo6. A competitive immunoassay was optimized in the indirect ELISA format, and the hapten density of antigens was revealed as an important factor to be studied. Finally, the developed assay was applied to the analysis of PC in fortified soybean sprouts as a proof of

concept. Further studies are being carried out with other oilseed samples and cereals, including samples from the market, which will be used to comprehensively validate the described immunochemical assay.

Acknowledgements

This work was supported by *Ministerio de Educación y Ciencia* (AGL2006-12750-C02-01/02/ALI) and cofinanced by FEDER funds. J.P. was hired by *Consejo Superior de Investigaciones Científicas* (CSIC) under a predoctoral *I3P* contract of *Ministerio de Ciencia e Innovación* and the European Social Fund. F.A.E.T. and J.V.M. were hired by CSIC with postdoctoral contracts, the former under the *Juan de la Cierva* Program and the latter under the *Ramón y Cajal* Program, both cofinanced by *Ministerio de Ciencia e Innovación* and by the European Social Fund. We also thank Ana Izquierdo-Gil and Laura López-Sánchez for excellent technical assistance.

Limited amounts of the described immunoreagents are available upon request.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/.

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Footnotes

¹ **Abbreviations used:** ELISA, enzyme-linked immunosorbent assay; PC, picoxystrobin; pAb, polyclonal antibody; cELISA, competitive ELISA; rt, room temperature; HRMS, high resolution mass spectrometry; EI, electron-impact; HRP, horseradish peroxidase; OVA, ovalbumin; GAR, goat anti-rabbit immunoglobulin; BSA, bovine serum albumin; FBS, fetal bovine serum; DMF, *N,N*-dimethylformamide; THF, tetrahydrofuran; LDA, lithium diisopropylamide; DSC, *N,N'*-disuccinimidyl carbonate; MR, molar ratio; LOD, limit of detection; LOQ, limit of quantification. For buffer name abbreviations and composition see the Materials and Methods section.

Figure legends

Fig. 1

Synthesis of hapten PCb6.

Fig. 2

Synthesis of hapten PCo6.

Fig. 3

One of the most stable conformations of PC, calculated using CONFLEX with MM3 molecular mechanics to systematically search for low energy conformers (CACHeworksystem Pro Version 7.5.0.85). The elements are represented in the following manner: carbon, grey; oxygen, red; nitrogen, blue; fluorine, green. The arrows point out the attachment sites of the C6 hydrocarbon spacer arm of haptens PCa6, PCb6, and PCo6.

Fig. 4

Influence of solvent concentration over the signal (A_{\max}) and detectability (IC_{50}) of the competitive assay with pAb rPCo6#1 and the heterologous coating conjugate OVA–PCa6 (MR = 3). Values are the mean of four independent determinations.

Fig. 5

Overlaid contour plots of the variation (%) of A_{\max} and IC_{50} values with respect to buffer pH and ionic strength (I). The white area sets the limits of acceptable pH and I conditions.

Fig. 6

Standard inhibition curves for PC obtained by cELISA in two buffer systems with equivalent ionic strength but different phosphate concentration, 10 mM (triangles) and 100 mM

(circles), using plates coated with OVA-PCa6 (MR = 3) at 1000 ng/mL and antiserum rPCo6#1 diluted 1/10000. Values are the mean of three independent experiments. The A_{max} values were between 0.8 and 1.2, the slopes were about -0.9, and the lower asymptotes were below 15% of the A_{max} .

Fig. 7

Matrix interferences produced by QuEChERS-based soybean sprout extracts over the inhibition curve of the rPCo6#1 conjugate-coated cELISA.

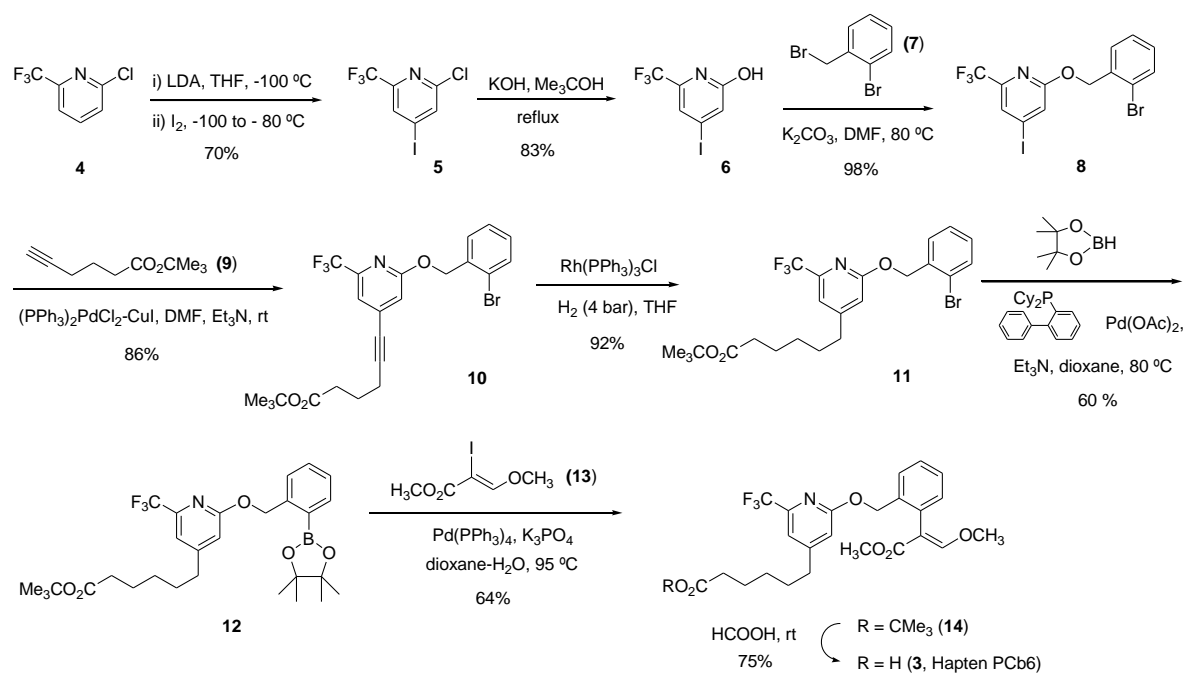


Figure 1, Parra et al.

Figure

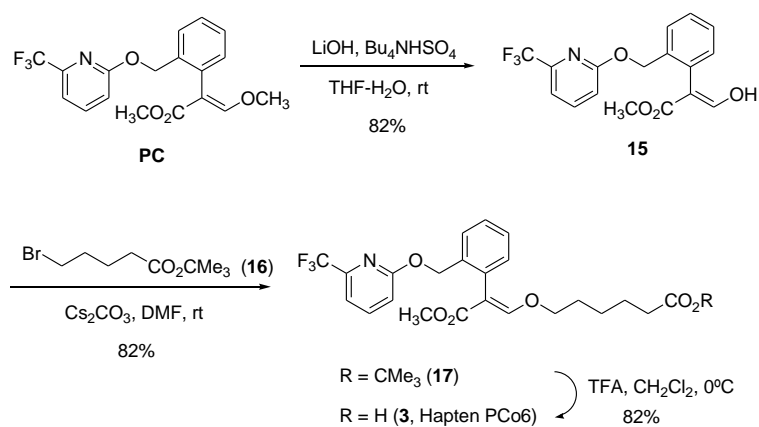


Figure 2, Parra et al.

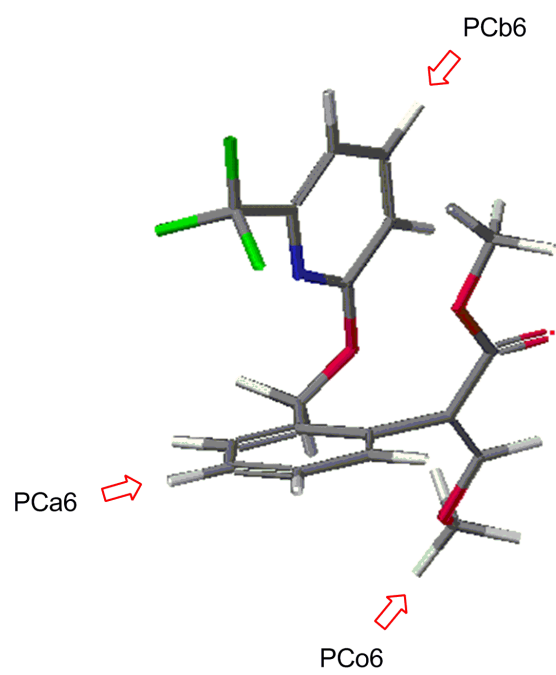


Figure 3, Parra et al.

Figure

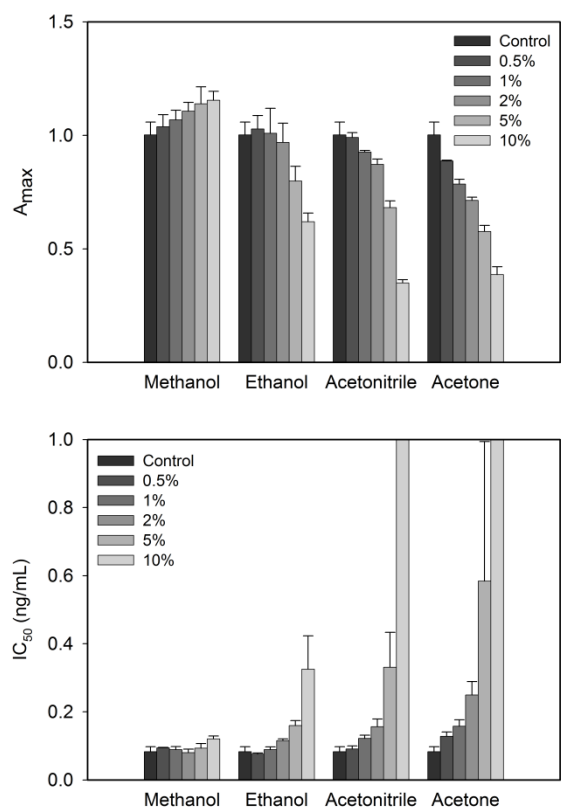


Figure 4, Parra et al.

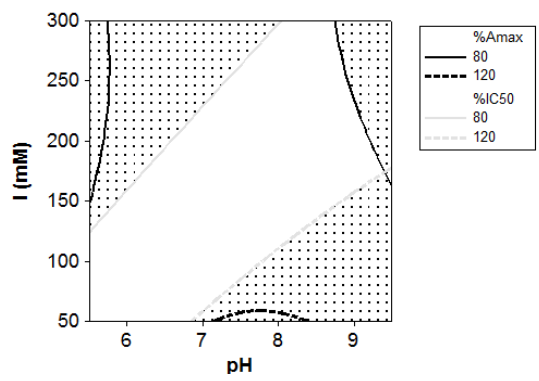


Figure 5, Parra et al.

Figure

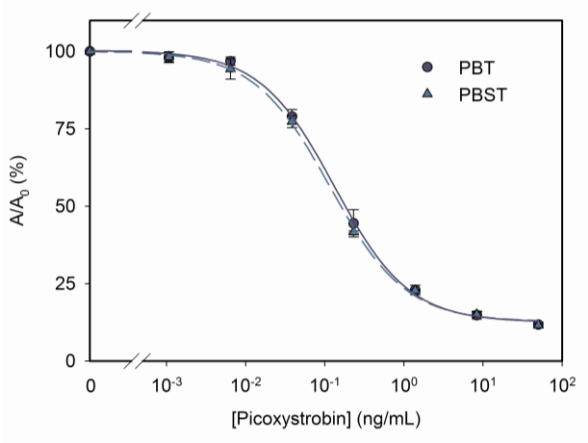


Figure 6, Parra et al.

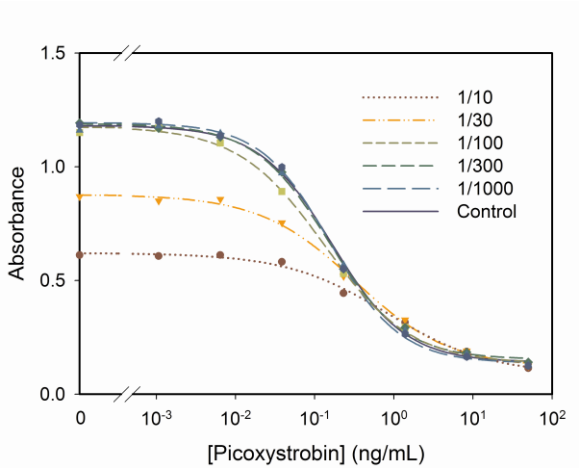
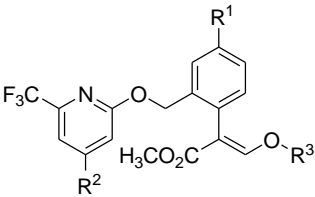


Figure 7, Parra et al.

Table 1
Structure of PC and the synthetic haptens



| | R ¹ | R ² | R ³ |
|----------|---|---|---|
| PC | H | H | CH ₃ |
| PCa6 (1) | (CH ₂) ₅ CO ₂ H | H | CH ₃ |
| PCb6 (2) | H | (CH ₂) ₅ CO ₂ H | CH ₃ |
| PCo6 (3) | H | H | (CH ₂) ₅ CO ₂ H |

Table 2

Characterization of anti-PC pAbs by checkerboard direct competitive assays^a.

| Immunizing hapten | HRP tracer | Rabbit #1 | | | | Rabbit #2 | | | |
|----------------------|---------------|-----------------------------------|---------------------|----------------|-----------------------------|-----------------------------------|---------------------|------------|-----------------------------|
| | | pAb dilution ($\times 10^3$) | [Tracer] (ng/mL) | A_{\max} | IC ₅₀ (ng/mL) | pAb dilution ($\times 10^3$) | [Tracer] (ng/mL) | A_{\max} | IC ₅₀ (ng/mL) |
| PCa6 | PCa6 | 1 | 1000 | 1.02 | 36.88 | 30 | 30 | 1.09 | 2.50 |
| | PCb6 | 1 | 1000 | - ^b | - | 1 | 1000 | - | - |
| | PCo6 | 1 | 1000 | - | - | 1 | 100 | 0.98 | 1.40 |
| PCb6 | PCa6 | 1 | 1000 | - | - | 1 | 1000 | - | - |
| | PCb6 | 30 | 30 | 1.07 | 1.51 | 10 | 300 | 0.84 | 2.83 |
| | PCo6 | 1 | 1000 | - | - | 1 | 1000 | - | - |
| PCo6 | PCa6 | 1 | 1000 | 1.38 | 0.15 | 1 | 300 | 0.86 | 3.05 |
| | PCb6 | 1 | 1000 | - | - | 1 | 1000 | - | - |
| | PCo6 | 3 | 300 | 0.84 | 0.48 | 3 | 100 | 0.81 | 0.62 |

^a A capture antibody solution was employed at 3000 ng/mL. ^b The maximum signal was below 0.8 under these conditions.

Table 3

Characterization of anti-PC pAbs by checkerboard indirect competitive assays.

| Immunizing hapten | OVA conjugate | Rabbit #1 | | | | Rabbit #2 | | | |
|----------------------|------------------|-----------------------------------|----------------------|------------|-----------------------------|-----------------------------------|----------------------|------------|-----------------------------|
| | | pAb dilution ($\times 10^3$) | [Coating] (ng/mL) | A_{\max} | IC ₅₀ (ng/mL) | pAb dilution ($\times 10^3$) | [Coating] (ng/mL) | A_{\max} | IC ₅₀ (ng/mL) |
| PCa6 | PCa6 | 10 | 1000 | 1.32 | 17.04 | 10 | 100 | 1.22 | 9.46 |
| | PCb6 | 3 | 1000 | 1.36 | - ^a | 3 | 100 | 1.07 | - |
| | PCo6 | 3 | 1000 | 1.41 | 1.91 | 30 | 1000 | 1.34 | 2.46 |
| PCb6 | PCa6 | 3 | 1000 | 1.35 | 1.80 | 10 | 1000 | 1.25 | 1.40 |
| | PCb6 | 30 | 100 | 1.00 | 1.18 | 30 | 100 | 0.91 | 1.07 |
| | PCo6 | 10 | 1000 | 1.50 | 2.53 | 30 | 1000 | 1.09 | 1.18 |
| PCo6 | PCa6 | 10 | 1000 | 1.25 | 0.25 | 30 | 1000 | 0.88 | 3.53 |
| | PCb6 | 10 | 1000 | 1.47 | 2.68 | 30 | 1000 | 0.99 | 1.10 |
| | PCo6 | 10 | 100 | 1.09 | 0.48 | 10 | 100 | 0.93 | 0.81 |

^a No inhibition was observed.

Table 4

Effect of the hapten-to-protein MR of OVA–PCa6 conjugate over the inhibition curve parameters obtained with pAb rPCo6#1.

| OVA–PCa6 conjugate | [Coating] (ng/mL) | rPCo6#1 dilution ($\times 10^3$) | A_{\max} | Slope | IC_{50} (ng/mL) | A_{\min} |
|--------------------|----------------------|---------------------------------------|------------|-------|----------------------|------------|
| MR 0.6 | 100 | 1 | 0.37 | 1.36 | 0.32 | 0.27 |
| | 300 | 1 | 0.66 | 1.50 | 0.25 | 0.30 |
| | 1000 | 3 | 0.67 | 1.16 | 0.13 | 0.19 |
| MR 3.0 | 100 | 1 | 0.96 | 1.31 | 0.42 | 0.27 |
| | 300 | 3 | 0.95 | 1.09 | 0.15 | 0.16 |
| | 1000 | 10 | 1.01 | 1.06 | 0.10 | 0.13 |
| MR 6.0 | 100 | 1 | 1.42 | 1.51 | 0.47 | 0.23 |
| | 300 | 3 | 0.81 | 1.27 | 0.40 | 0.25 |
| | 1000 | 10 | 1.39 | 1.08 | 0.29 | 0.15 |

Table 5
Recoveries from PC-spiked soybean sprouts by
the proposed cELISA procedure (% \pm s, n=3).

| Spiked PC ($\mu\text{g/kg}$) | Dilution | | |
|-----------------------------------|--------------|----------------|--------------|
| | 1/100 | 1/300 | 1/1000 |
| 5 | 100 \pm 11 | - ^a | - |
| 10 | 106 \pm 13 | 108 \pm 15 | - |
| 50 | 110 \pm 10 | 118 \pm 19 | 104 \pm 3 |
| 100 | 97 \pm 8 | 117 \pm 19 | 114 \pm 13 |
| 500 | - | 98 \pm 7 | 117 \pm 13 |
| 1000 | - | - | 112 \pm 19 |

^a Out of the linear range.